Pathogenesis of H5N1 Influenza Virus Infections in Mice and Ferret Models Differs According to Respiratory Tract or Digestive System Exposure

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Background. Epidemiologic, clinical, and laboratory data suggest that H5N1 influenza viruses are transmitted through and predominantly affect the respiratory system of mammals. Some data suggest digestive system involvement. However, direct evidence of alimentary transmission and infection in mammals is lacking.

Methods. Infection with and pathogenesis of 4 H5N1 viruses were assessed in mice and ferrets inoculated intranasally or intragastrically with virus in liquid. In addition, ferrets were fed infected raw chicken meat or minced meat administered into the stomach by gavage with a tube.

Results. Only one virus, A/Whooper swan/Mongolia/244/05, was able to infect mice after intragastric inoculation in liquid, whereas no evidence of infection was observed in ferrets after intragastric inoculation. Consumption of infected meat by ferrets resulted in respiratory system infection only (due to A/Muscovy duck/Vietnam/209/05 and A/Whooper swan/Mongolia/244/05 viruses) or in both severe respiratory and systemic infection with predominant involvement of the liver, pancreas, and large and small intestine (due to A/Vietnam/1203/04 virus). Direct intragastric exposure to infected meat (A/Vietnam/1203/04 virus) resulted in lethal systemic disease mainly affecting the intestine, liver, and pancreas but not involving the lungs.

Conclusions. Our results demonstrated that exposure of the digestive system to H5N1 influenza viruses could initiate infection either through the tonsil, with spread to respiratory tissues, or through intestinal infection, with spread to the liver and pancreas.

Highly pathogenic avian influenza (HPAI) viruses of the H5N1 subtype are zoonotic agents that continue to pose a threat to both veterinary and public health [1, 2]. In late 2003, these H5N1 HPAI viruses expanded their geographic range outside of southern China, initiating unprecedented epizootics in poultry and sporadic cases of disease in humans in eastern and southeastern Asia [3, 4]. Between 2003 and mid-2008, outbreaks of infection with H5N1 HPAI viruses were reported in poultry and/or wild birds in 60 countries in Asia, Europe, and Africa, and cases of human disease for which case-fatality rates were high were reported from 15 of these countries [5–8]. The endemicity of H5N1 HPAI viruses in poultry in villages in Eurasia and Africa [9] continues to provide conditions favoring bird-to-human transmission and possible emergence of pandemics.

To date, human-to-human transmission of H5N1 HPAI viruses has been very limited, and most cases of infection in humans have occurred through close contact with infected live or dead poultry [1, 2]. A few cases in humans have implicated direct oral contact either through consumption of uncooked blood from infected ducks and raw poultry products [10] or through oral exposure from sucking exudate from the upper respiratory tract of infected fighting cocks [11]. Large felids

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(tigers and leopards) in zoos in Thailand became infected after being fed chickens infected with H5N1 HPAI virus [12, 13], and, in experiments in domestic cats, H5N1 HPAI virus was transmitted after the cats were fed infected chickens or chicken meat [14, 15]. The researchers in the cat study proposed that H5N1 HPAI viruses could infect the host via the digestive system, but they did not rule out the possibility that pharyngeal exposure during consumption of infected chickens produced upper respiratory tract infection, followed by systemic infection, as the pathogenic mechanism for intestinal autonomic nerve infection [15]. Studies of the transmission of H5N1 HPAI viruses in a ferret model have shown negligible transmissibility via respiratory droplets [16] and inefficient but repeatable transmission via direct contact [17], although whether the site of initiation of the infection was respiratory or digestive is not clear.

In the present study, mice and ferrets were intranasally and intragastrically inoculated with 4 H5N1 HPAI viruses representing major phylogenetic and antigenic clades of H5 hemagglutinin (HA) [18]. Ferrets were also exposed to H5N1 viruses through oral consumption or gastric gavage of infected chicken meat. We demonstrated that infection can be initiated either through the respiratory system or the digestive system and that the site of initial replication influences pathogenesis of the disease.

MATERIALS AND METHODS

Viruses and cells. Four H5N1 HPAI viruses representing major phylogenetic and antigenic clades of H5 HA were used: clades 2.1 and 2.2 (A/Chicken/Indonesia/7/03 [Ck/Indo/03] virus and A/Whooper swan/Mongolia/244/05 [WS/Mong/05] virus, respectively) (provided by D.E.S., Southeast Poultry Research Laboratory (SERPL), Athens, Georgia), clade 1 (A/Vietnam/1203/04 [VN/04] virus) (isolated by Nguyen Tran Hien, National Institute of Hygiene and Epidemiology, Hanoi, Vietnam; provided by Kanta Subbarao, National Institutes of Health, Bethesda, Maryland), and clade 2.3 (A/Muscovy duck/Vietnam/209/05 [MDk/VN/05] virus) (provided by Nguyen Van Cam, National Center for Veterinary Diagnosis, Hanoi, Vietnam). Virus stocks were produced by passage in 10-day-old embryonating chicken eggs.

Madin-Darby canine kidney (MDCK) cells (American Type Culture Collection) were cultured in Dulbecco's modified Eagle medium supplemented with 5% fetal bovine serum. The $TCID_{50}$ was determined for MDCK cells after incubation at 37°C for 3 days, and the 50% egg infectious dose (EID_{50}) was determined in 10-day-old embryonating chicken eggs. $TCID_{50}$ and EID_{50} values were calculated using the Reed-Muench method [19].

All experiments involving H5N1 viruses were performed in a biosafety level 3–enhanced agriculture high-biocontainment facility. All personnel were required to use respiratory protection when working with live viruses or infected animals.

Infection of mice. Female BALB/c mice (age, 7–8 weeks old) (Simonsen Laboratories), assigned to groups of 15, were anesthetized by isoflurane inhalation and were inoculated either intranasally (50 μL/mouse) or intragastrically (by gavage with the use of a needle; 100 μL/mouse) with 10^3 EID_{50} of virus in PBS. Sham control mice were inoculated intranasally or intragastrically with PBS alone. Mice were monitored for clinical signs and survival, and body weight was measured daily during the 15-day observation period. Four mice from each group were euthanized on the fifth day post inoculation (dpi), and organ samples were harvested for virus titration and histologic examination.

Infection of ferrets. Eight groups of four 6- to 8-month-old female outbred ferrets (Marshal Farm) were anesthetized by isoflurane inhalation or by intramuscular injection of ketamine (25 mg/kg of body weight) and xylazine (3 mg/kg of body weight), and were inoculated either intranasally (0.5 mL/ferret) or intragastrically (by gavage with the use of a tube; 2.0 mL/ferret) with 10^6 EID_{50} of virus in PBS. Three groups of 4 ferrets were fed 30 g of meat (virus dose, $\sim 10^{9.5}$ EID_{50}/ferret) collected from chickens infected with VN/04, WS/Mong/05, and MDk/VN/05 viruses by use of a method described elsewhere [20]. An additional group of 4 ferrets received 2 g of minced meat from chickens infected with VN/04 virus ($10^{8.3}$ EID_{50}/ferret) through a stomach gavage tube while they were anesthetized with ketamine and xylazine.

For 15 days, ferrets were monitored daily for clinical signs and survival. Body temperature was recorded daily by use of implanted electronic transponders, and body weight was measured at 0 and 1 dpi and on alternating days until 11 dpi. To monitor virus shedding, nasal washes and rectal swab specimens were collected, as described elsewhere [17, 21], at 1 dpi and on alternate days until 9 dpi. Two ferrets (albeit only those ferrets that consumed meat infected with WS/Mong/05 virus) from each group were humanely euthanized at 2 dpi and at 5 dpi, and organ and tissue samples were collected for virus titration and histologic examination.

All studies involving animals were conducted under applicable laws and guidelines and after approval was received from the SERPL institutional animal care and use committee.

Virus titration in animal samples. Tissues collected from mice and ferrets were weighed and homogenized in sterile PBS with antibiotics to obtain ~10% (w/v) homogenates. Virus titers in MDCK cells were determined in tissue homogenates, nasal washes, and rectal swab specimens and were expressed as the $\log_{10}$ $TCID_{50}$ value per gram of tissue or as the $TCID_{50}$ per milliliter of nasal wash or rectal swab media.

Histologic analysis and immunohistochemical analysis (IHC). Formalin-fixed and paraffin-embedded tissue sections were stained with hematoxylin-eosin (HE), for histologic evaluation, or with a mouse-derived monoclonal antibody (P13C11) specific for type A influenza virus nucleoprotein antigen as the pri-

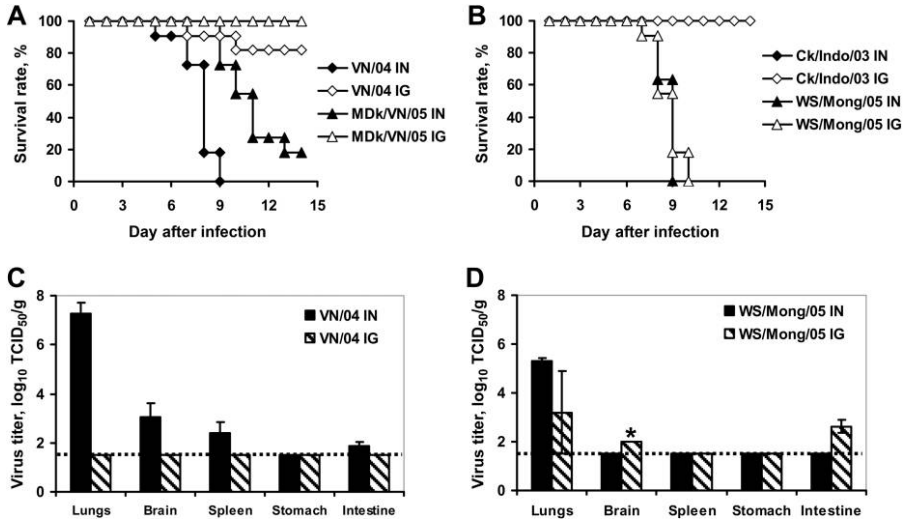

Figure 1. Survival rates *(A, B)* of and virus titers *(C, D)* in mice after intranasal (IN) and intragastric (IG) inoculation with H5N1 influenza viruses. Fifteen mice (14 of which were in the group receiving IG inoculation with A/Chicken/Indonesia/7/03 [Ck/Indo/03] virus) were inoculated with H5N1 viruses. Four mice from each experimental group were humanely killed on the fifth day post infection (dpi), to collect tissue samples, and the rest of the animals were observed for survival over a 15-day period. Virus titers in tissue homogenates were determined in Madin-Darby canine kidney (MDCK) cells. The results are expressed as the average ($\pm$SD) of the $\log_{10}$ $TCID_{50}$ value per milliliter. The lower limit of detection was $10^{1.5}$ $TCID_{50}$ per gram. *Virus detected in 1 mouse.

mary antibody. The procedures used to perform IHC on ferret tissues followed those described elsewhere [22, 23]. Fast Red was used as the substrate chromagen, and slides were counterstained with hematoxylin. For the mice tissues, the M.O.M. Kit (Vector Laboratories) was used for the detection of the primary antibody, as per the manufacturer's instructions. The AEC-Peroxidase Substrate Kit (Vector Laboratories) was used as the substrate chromagen, and hematoxylin was used as a counterstain.

Serologic assays. Serum samples obtained from ferrets before exposure were tested in a hemagglutination inhibition (HI) assay [24] with inactivated antigens for challenge H5N1 viruses and currently circulating human influenza A and B viruses. Seroconversion was assessed by determining the neutralizing antibody titers against the challenge viruses in the preexposure (ferrets only) and postexposure (both ferrets and mice) serum samples collected before infection and at 15 dpi, respectively, by means of virus neutralization (VN) in MDCK cells, as described elsewhere [25].

RESULTS AND DISCUSSION

Virus pathogenicity and organ tropism in a mouse model. To determine whether H5N1 HPAI viruses could infect mammalian hosts through either respiratory or digestive systems, virus infectivity and pathogenesis were assessed in mice inoculated with virus in liquid media administered intranasally or intragastrically. Intranasal inoculation with MDk/VN/05, VN/04, and WS/Mong/05 viruses resulted in severe disease with associated mortality rates of 80%–100%, whereas no clinical signs or deaths were observed in association with intranasal inoculation of Ck/Indo/03 virus (figure 1*A* and 1*B*). By contrast, only intragastrically inoculated WS/Mong/05 virus produced a high mortality rate (100%) (figure 1*B*), whereas VN/04 virus produced a mortality rate of $<$20% and MDk/VN/05 and Ck/Indo/03 viruses did not produce clinical signs or death (figure 1*A* and 1*B*).

On the basis of findings for tissues collected at 5 dpi, the severity and pathogenesis of infection varied with individual virus strains and the route of inoculation. Intranasal inoculation with VN/04 and WS/Mong/05 viruses caused systemic infection with virus isolation from or detection in the lungs, brain, spleen, and intestine, as well as lesions associated with bronchitis, bronchiolitis, alveolitis, encephalitis, and splenic lymphoid depletion (figures 1*C* and 1*D* and table 1). Similarly, intragastric inoculation with WS/Mong/05 virus produced systemic infection with virus recovery or antigen detection in the lungs, brain, spleen, stomach, and intestine, but intragastric inoculation of VN/04 virus did not produce lesions or infection (figure 1*C* and 1*D* and table 1). Mice intragastrically inoculated with WS/Mong/05 virus had mild enteritis and gastritis with infrequent viral antigen staining in the lamina propria of the submucosa, and such lesions were absent in mice intranasally inoculated with WS/Mong/05 virus, although antigen was rarely identified. Intranasal inoculation with MDk/VN/05 virus resulted in high-titer virus recovery or antigen detection in the lungs and brain, and it was accompanied by moderate lung and brain lesions, whereas intragastric inoculation did not produce lesions or infection (tables 1 and 2) (data not shown). Intranasal or intragastric inoculation with Ck/Indo/03 virus did not produce lesions, and viral

Table 1. Distribution of histologic lesions and viral antigen after intranasal (IN) and intragastric (IG) inoculation of mice with H5N1 highly pathogenic avian influenza viruses.

Virus, route of inoculation	Histologic examination[a]/ IHC[b] findings, by tissue				
	Lungs	Brain	Spleen	Stomach	Intestine
Ck/Indo/03					
IN	−/−	−/−	−/−	−/−	−/−
IG	−/−	−/−	−/−	−/−	−/−
VN/04					
IN	+++/+++	++/++	+/+	−/−	−/−
IG	−/−	−/−	−/−	−/−	−/−
WS/Mong/05					
IN	+++/+++	+/+	++/+	−/+	−/+
IG	++/++	+/+	++/+	+/+	+/+
MDk/VN/05					
IN	++/++	++/++	−/−	−/−	−/−
IG	−/−	−/−	−/−	−/−	−/−

NOTE. Ck/Indo/03, A/Chicken/Indonesia/7/03; IHC, immunohistochemical analysis; MDk/VN/05, A/Muscovy duck/Vietnam/209/05; VN/04, A/Vietnam/1203/04; WS/Mong/05, A/Whooper swan/Mongolia/244/05.

[a] −No lesions. +Mild lesions. ++Moderate lesions. +++Severe lesions.

[b] −No antigen staining. +Infrequent staining. ++Common staining. +++Widespread staining.

antigen was not detected, although virus was recovered from the lungs, and seroconversion was identified in intranasally inoculated mice (tables 1 and 2) (data not shown).

In these mouse experiments, only WS/Mong/05 virus was infective and lethal after both intranasal and intragastric inoculation, producing systemic disease. However, the isolation of virus from the intestine and the presence of gastrointestinal lesions with accompanying viral antigen implied that intragastric inoculation produced a unique pathogenic phase in the digestive system, followed by disseminated systemic infection; however, intranasal inoculation produced minimal evidence for digestive system involvement. For the other 3 viruses, intranasal inoculation initiated infection through the respiratory tract and culminated as asymptomatic respiratory infection (Ck/Indo/03 virus), fatal respiratory and neurologic disease (MDk/VN/05 virus), or fatal systemic disease (VN/04 virus); however, intragastric inoculation did not initiate an infection process. Although the 4 mice that were intragastrically inoculated with VN/04 virus and had samples collected at 5 dpi lacked evidence of infection, deaths did occur in 2 of 11 mice in the pathotyping test. Such deaths may have resulted from pharyngeal/upper respiratory tract exposure when the gastric inoculation cannula was withdrawn. VN/04 virus is extremely lethal in mice (mean mouse lethal dose [MLD_{50}], 1.6 EID_{50} or 0.6 plaque-forming units) [26, 27], and even a negligible amount of virus in the pharynx could initiate upper respiratory tract infection leading to fatal systemic disease.

Virus pathogenicity and organ tropism in ferrets. To further evaluate the ability of H5N1 HPAI viruses to infect mammals through the digestive tract, 4 viruses were studied in a ferret model. Ferrets are one of the best small-mammal models for studies of H5N1 HPAI virus infection, and natural H5N1 HPAI infections have been observed in a close relative, the stone marten *(Martes foina)* [28], which is in the same family, Mustelidae. Ferrets are naturally susceptible to infection with human and avian influenza A viruses, and pathogenesis of H5N1-induced disease in ferrets resembles, in many characteristics, that described for H5N1 infection in humans [21, 27, 29–31].

Ferrets used in the present study were determined to be seronegative for circulating influenza B viruses and H1N1 and H5N1 influenza A viruses, by use of the HI test. However, all animals possessed recognizable HI antibody titers (1:320 to 1:640) to human H3N2 influenza A virus (A/Hiroshima/52/05 virus).

Two H5N1 viruses, Ck/Indo/03 and MDk/VN/05, had low pathogenicity in ferrets after intranasal inoculation, producing mild, almost asymptomatic respiratory disease with slight weight loss and fever at 1–3 dpi (data not shown), presence of H5 VN antibodies in all postinfection serum samples (table 3), and infrequent, low-titer virus recovery from nasal washes (data not

Table 2. Serum neutralizing antibody titers to H5N1 highly pathogenic avian influenza viruses in mice after exposure.

	Serum neutralizing titer, by virus and inoculation route							
	MDk/VN/05		VN/04		Ck/Indo/03		WS/Mong/05	
Mouse group	IN	IG	IN	IG	IN	IG	IN	IG
Virus inoculated	80–160	<20[a]	ND	<20	40–80	<20	ND	ND
Sham control	<20	<20	<20	<20	<20	<20	<20	<20

NOTE. Serum samples from blood obtained from mice that survived to 15 days post infection (dpi) were pooled in groups of 3 and tested. The number of mice that received intranasal (IN) and intragastric (IG) inoculation, respectively, were as follows: in the A/Muscovy duck/Vietnam/209/05 (MDk/VN/05) virus group, 2 and 11 mice; in the A/Chicken/Indonesia/7/03 (Ck/Indo/03) virus group, 11 and 10 mice; and in the A/Vietnam/1203/04 (VN/04) virus group, 0 and 9 mice. In addition, 11 sham control mice received IN or IG inoculation with sterile PBS. ND, 100% mortality rate noted; WS/Mong/05, A/Whooper swan/Mongolia/244/05 virus.

[a] Titers of <20 were considered to denote negative findings.

Table 3. Serum neutralizing antibody titers to H5N1 highly pathogenic avian influenza (HPAI) viruses in ferrets before and after exposure.

Virus group, route of exposure	Neutralizing antibody titer	
	Before exposure	After exposure
Ck/Indo/03		
IN	<20	80–160
IG	<20	<20
VN/04		
IN	<20	ND
IG	<20	<20
Meat	<20	ND
Meat IG	<20	<20[a]; ND
MDk/VN/05		
IN	<20	160–320
IG	<20	<20
Meat	<20	160–320
WS/Mong/05		
IN	<20	1280–2560
IG	<20	<20
Meat	<20	ND

NOTE. Ck/Indo/03, A/Chicken/Indonesia/7/03 virus; MDk/VN/05, A/Muscovy duck/Vietnam/209/05 virus; Meat, virus-infected meat consumed orally; Meat IG, virus–infected meat that was minced and intragastrically administered to ferrets by gavage with the use of a tube; WS/Mong/05, A/Whooper swan/Mongolia/244/05 virus; VN/04, A/Vietnam/1203/04 virus. ND, animals died or were euthanized because of severe disease symptoms (VN/04 IN and VN/04 Meat groups), or they were humanely killed for tissue sampling on day 2 and 5 (WS/Mong/05 Meat group).

[a] Determined for a serum sample from 1 animal.

shown). Ferrets intranasally inoculated with MDk/VN/05 virus had mild bronchitis and minimal alveolitis without detectable AI viral antigen, whereas ferrets inoculated with Ck/Indo/03 virus lacked histologic lesions and viral antigen. By contrast, intragastric inoculation of ferrets with Ck/Indo/03 and MDk/VN/05 viruses in liquid media did not result in infection, as was evident by the lack of (1) clinical signs, (2) virus in nasal washes, rectal swab specimens, and internal tissues, and (3) VN antibodies in postexposure serum samples (table 3). However, consumption of meat from chickens infected with MDk/VN/05 virus resulted in mild respiratory disease, recovery of virus from nasal washes, and detection of VN antibodies in serum samples, similar to the results observed after intranasal inoculation. Thus, the site for initial virus infection after ingestion of infected meat did not suggest the gastrointestinal tract but, rather, the respiratory tract, most likely as a result of pharyngeal contact during consumption, followed by extension into the upper respiratory tract.

Intranasal inoculation of WS/Mong/05 virus was moderately pathogenic, resulting in severe respiratory disease accompanied by fever on days 1–3 (data not shown), 10% loss in body weight, (figure 2*A*), moderate bronchointerstitial pneumonia, and development of VN antibodies (table 3). WS/Mong/05 virus was recovered from nasal washes (figure 2*C*) and replicated in respiratory tissues (with the highest titers noted in the nasal turbinate and lungs) and tonsils; it was also detected in the olfactory bulbs of 1 ferret (figure 3*A*). Feeding ferrets WS/Mong/05 virus–infected chicken meat resulted in respiratory disease that was milder than that noted in intranasally inoculated animals (figure 2*A*) and included bronchointerstitial pneumonia. Virus was re-

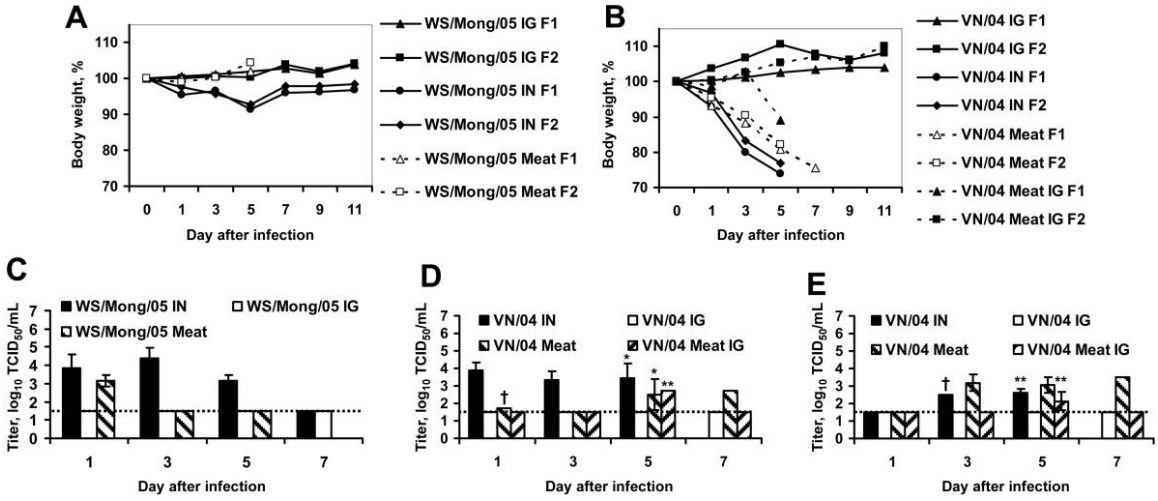

Figure 2. Changes in body weight *(A, B)* of and in the virus titers in nasal washes *(C, D)* and rectal swab specimens *(E)* from ferrets exposed to A/Whooper swan/Mongolia/244/05 (WS/Mong/05) *(A, C)* and A/Vietnam/1203/04 (VN/04) *(B, D, E)* H5N1 influenza viruses by different routes. Virus titers were determined in Madin-Darby canine kidney cells and were expressed as the average values (±SD) of $\log_{10}$ $TCID_{50}$ per milliliter of nasal wash or swab media. The lower limit of detection was $10^{1.5}$ $TCID_{50}$ per milliliter. *Virus detected in 3 ferrets. **Virus detected in 2 ferrets. †Virus detected in 1 ferret. F1, ferret 1; F2, ferret 2; Meat, virus-infected meat consumed orally; Meat IG, virus–infected meat that was minced and intragastrically administered to ferrets by gavage with the use of a tube.

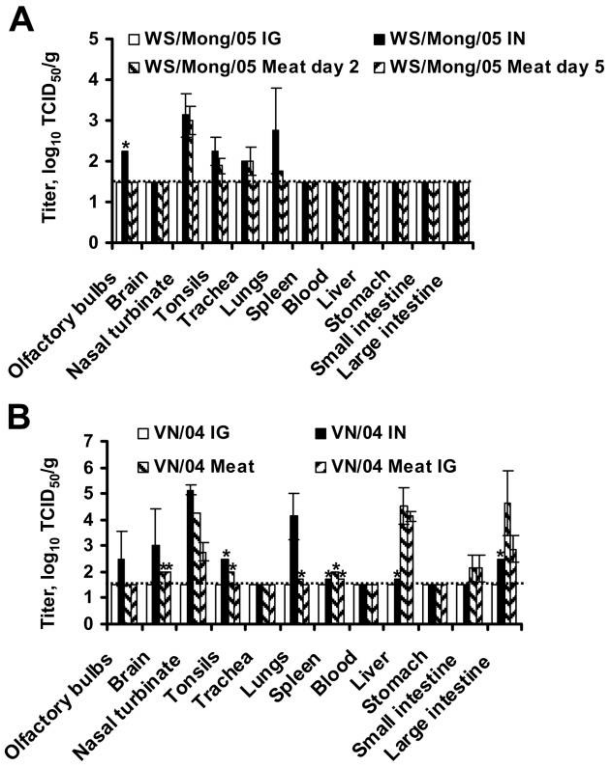

Figure 3. Titers of A/Whooper swan/Mongolia/244/05 (WS/Mong/05) *(A)* and A/Vietnam/1203/04 (VN/04) *(B)* H5N1 influenza viruses in ferret organs. Virus titers in 10% tissue homogenates were determined in Madin-Darby canine kidney cells. The results are presented as the average values (±SD) of the $\log_{10}$ $TCID_{50}$ per milliliter. The lower limit of detection was $10^{1.5}$ $TCID_{50}$ per gram of tissue. *Virus detected in 1 ferret. Meat, virus-infected meat consumed orally; Meat IG, virus–infected meat that was minced and intragastrically administered to ferrets by gavage with the use of a tube.

covered from nasal washes at 1 dpi only (figure 2*C*), and virus was detected in the respiratory organs and tonsils of ferrets at 2 dpi, with the highest virus titers noted in nasal turbinate. Tissues harvested at 5 dpi did not contain infective virus (figure 3*A*). The predominance of respiratory data suggests that intranasal exposure or oral consumption of infected meat initiated virus infection in the upper respiratory tract similar to that of MDk/VN/05 virus—that is, intranasal inoculation with direct replication in the nasal cavity and local extension into the olfactory bulbs during the consumption of infected meat most likely involved pharyngeal exposure with infection of the tonsils and extension to the tissues of the upper and lower respiratory tract. However, similar to Ck/Indo/03 and MDk/VN/05 viruses, WS/Mong/05 virus in liquid medium did not produce infection when intragastrically inoculated (figures 2 and 3 and table 3).

VN/04 virus was highly pathogenic after intranasal inoculation producing fever (data not shown); severe weight loss (figure 2*B*); neurologic signs; the death of 2 animals at 6 dpi; virus isolation from nasal and rectal swab specimens (figures 2*D* and 2*E*); and severe respiratory and systemic lesions, including bronchointerstitial pneumonia (figure 4*A*), encephalitis (figure 4*B*), and hepatic necrosis (data not shown). At 5 dpi, respiratory infection and systemic infection were predominant, with the highest titers noted in the nasal turbinates, lungs, brain, and olfactory bulbs (figure 3*B*); with high antigen content noted in the lungs, olfactory bulbs (figure 4*C*), brain, and liver (data not shown); and with low titers noted in the tonsils, spleen, and large intestine of 1 ferret (figure 3*B*). Similar to the other 3 H5N1 viruses, intragastric inoculation of VN/04 virus in liquid medium did not produce infection (figures 2, 3, and 4*D*; table 3). However, feeding ferrets infected meat resulted in clinical signs similar to those observed after intranasal inoculation of virus (figure 2*B*), and virus was recovered from nasal washes and rectal swab specimens (figures 2*D* and 2*E*). Although consumption of infected meat produced systemic infection, virus tropism and titers differed from those of intranasally inoculated animals. Ferrets fed infected meat had predominant digestive system infection evident as high viral titers in the liver and large intestine and as low titers in the small intestine (2 ferrets), and minor respiratory and systemic involvement was evident as low titers in the tonsil, spleen, lungs and brain of 1 ferret. These findings suggest that feeding infected meat could initiate infection via both the alimentary tract, leading to infection of the liver, and the pharynx and tonsil, leading to respiratory tract infection.

To determine whether direct exposure of the alimentary tract to infected meat could initiate virus infection in the alimentary tract, VN/04 virus–infected meat was minced and intragastrically administered to ferrets by gavage with use of a tube. Three of 4 inoculated ferrets developed lethal infection with systemic disease similar to that observed in ferrets that consumed infected meat (figure 2*B* and table 3). One survivor remained seronegative, indicating a lack of infection. At 5 dpi, virus was detected in both nasal washes and rectal swab specimens (figure 2*D* and 2*E*), and the virus was consistently isolated from both the large and small intestine. Virus was isolated from the liver in high titers, but it was inconsistently isolated from the spleen and brain in low titers. Lesions were predominantly noted in the digestive system and included severe cholangiohepatitis with bile duct epithelial necrosis, mild hepatocellular necrosis, and Kupffer cell proliferation. Viral antigen was observed in the bile duct epithelium, hepatocytes, and Kupffer cells (figures 4*E* and 4*F*); severe necrosis of pancreatic duct epithelium was accompanied by viral antigen in the necrotic epithelium (figure 4*G*); and mild crypt epithelial necrosis was noted in the duodenum (figure 4*H*). Lesions, virus, and viral antigen were lacking in the trachea, tonsils, olfactory lobes, and lung, but low virus titers were noted in the nasal cavity (figure 3*B*) (data not shown). Because intragastric administration of infected meat precluded exposure to the oropharynx, infection was initiated in the alimentary tract, as was evident by observations of intestinal virus replication and lesions, and it was spread through the liver into systemic circulation.

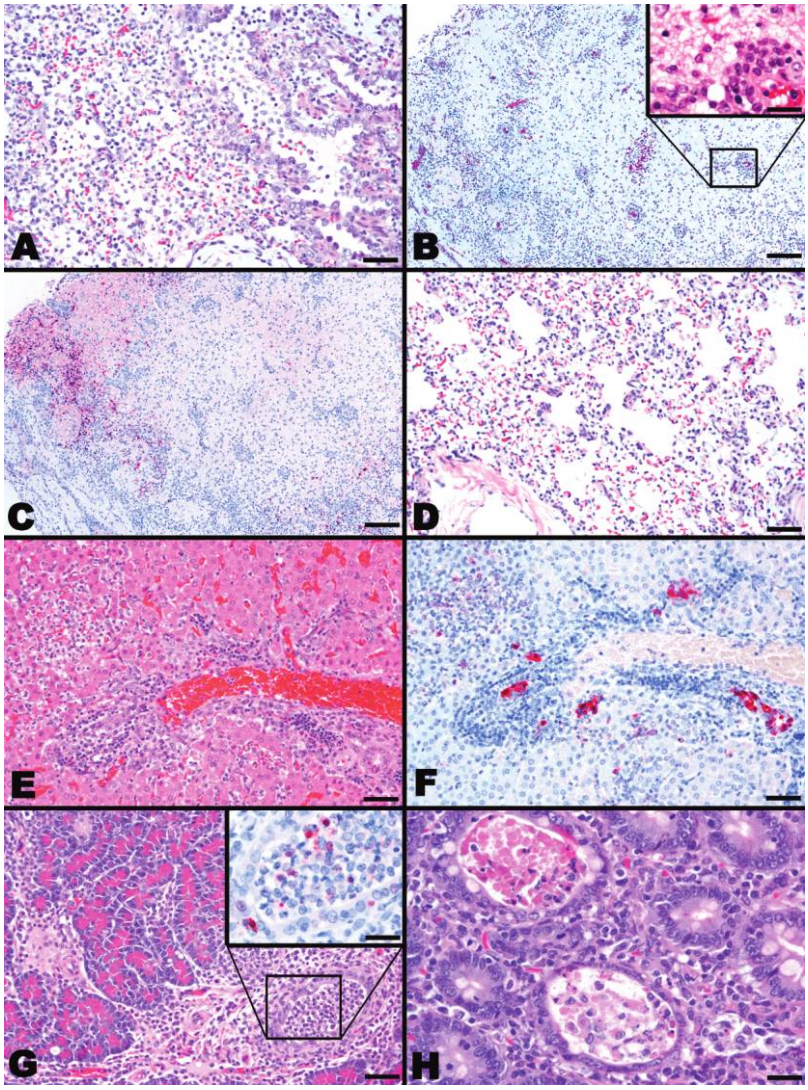

Figure 4. Histopathologic lesions and influenza viral antigen localization in ferrets infected with H5N1 highly pathogenic avian influenza (HPAI) virus A/Vietnam/1203/04 (VN/04) by different routes of exposure. *A,* Severe bronchointerstitial pneumonia with bronchiolar epithelial necrosis (after exposure by the intranasal [IN] route) at the fifth day post infection (dpi). The bar denotes 25 μm. *B,* Severe, diffuse malacia in the olfactory bulb with inflammatory cellular infiltration, moderate cavitation, and focal hemorrhage (after exposure by the IN route) at 6 dpi. The bar denotes 100 μm. Moderate mononuclear cellular infiltrates around perivascular areas, with necrosis of neurons and astrocytes *(insert).* The bar denotes 25 μm. *C,* Marked viral antigen localization in the neuropil, neurons, and astrocytes (after exposure by the IN route) at 6 dpi. The bar denotes 100 μm. *D,* Normal lung (after exposure by the intragastric [IG] route) at 5 dpi. The bar denotes 50 μm. *E,* Moderate cholangiohepatitis characterized by bile duct necrosis, mononuclear cellular infiltrate around the portal triad, and focal hepatocyte necrosis (after exposure by IG administration of infected meat) at 5 dpi. The bar denotes 50 μm. *F,* Viral antigen in the bile duct epithelia and hepatocytes (after exposure by IG administration of infected meat) at 5 dpi. The bar denotes 50 μm. *G,* Moderate necrosis of the interlobular pancreatic duct epitheliums with interluminal necrotic cellular debris and mononuclear cellular infiltrates (after exposure by IG administration of infected meat) at 5 dpi. The bar denotes 50 μm. Avian influenza viral antigen in the pancreatic duct epithelia and necrotic cellular debris *(insert).* The bar denotes 10 μm. *H,* Mild duodenal crypt epithelial cell degeneration and necrosis with intraluminal cellular debris (after exposure by IG administration of infected meat) at 5 dpi. The bar denotes 25 μm.

Human infection with H5N1 HPAI viruses has resulted in severe disease with predominant respiratory symptoms and lesions, and there have been only a few reports describing gastrointestinal symptoms and virus detection in and isolation from rectal swab specimens or fecal samples [1, 2, 31–33]. These clinical observations, together with epidemiologic data and findings from studies of animal models [10–15, 17], suggest that H5N1 virus infections could target not only the respiratory system but, also, the digestive system. In the present study, we demonstrated, in 2 mammalian models, that direct gastrointestinal exposure to virus or chicken meat containing virus could produce virus infection and lesions in the digestive system, including the small intestines, large intestines, liver, and pancreas, and that such infections could be fatal, depending on the virus strain and host.

However, the initial site of virus replication (the respiratory or digestive system) varied according to the virus strain, route of exposure, and medium. Oral consumption of infected meat and could lead to concurrent respiratory and digestive system infection, although respiratory infection was predominant.

With mice and ferrets, intranasal inoculation of virus in liquid initiated upper and lower respiratory tract infection, with local extension occurring via the olfactory nerves into the brain and, with some viruses, systemic infection. Similarly, respiratory infection followed by local extension into the brain or systemic infection has been reported for mice and ferrets after intranasal inoculation with a variety of H5N1 HPAI virus strains [16, 17, 21, 25–27, 29]. Direct exposure of virus in liquid by gavage was less successful as a route of exposure for producing infections in mice and ferrets (i.e., only WS/Mong/05 virus in mice produced infection and lesions, thus suggesting inactivation of the virus in the acidic gastric environment or the need for higher-exposure doses in the digestive system to produce infection, compared with respiratory exposure). However, exposure to H5N1 virus by either natural feeding or gastric gavage with infected chicken meat produced H5N1 virus infection in the ferrets. Most likely, the H5N1 virus within the meat was protected from inactivation during transit through the stomach to the intestines. The H5N1 virus is contained within the myocytes of infected chicken meat [20, 34].

With the 2 respiratory tropic viruses (MDk/VN/05 and WS/Mong/05) in ferrets, oral consumption exposed the pharynx to H5N1 virus, initiating tonsillar and upper respiratory tract infection but without evidence of digestive system involvement. However, with a highly pathogenic virus that produced severe systemic disease (VN/04), oral consumption initiated an upper respiratory infection; it was accompanied by intestinal infection and was spread to the liver possibly via portal or intestinal lymphatic systems. Gavage with minced, infected chicken meat confirmed this unique intestinal route of infection and a pathogenesis different from that noted for infection occurring after intranasal exposure. Natural cases of H5N1 virus infection in large felids, small felids, and a dog have been associated with the feeding or consumption of infected birds, resulting in predominantly severe lesions of pulmonary edema and hemorrhage and interstitial pneumonia [12, 13, 35, 36]; this finding suggests that the site of initiation of infection was the respiratory tract during feeding, possibly through direct pharyngeal and tonsillar infection or through inhalation of airborne virus generated during the ripping and tearing of carcasses associated with the eating process of carnivores. Similarly, the WS/Mong/05 H5N1 virus has been experimentally transmitted through the feeding of infected chicken meat to pigs and herring gulls [37, 38]. Although the pigs were asymptomatic, infection was initiated within and focused on the respiratory system, with virus isolated from or detected in tonsil, nasal turbinate, and nasal swab specimens but not in rectal swab specimens [37]. In the gulls, infection resulting from the consumption of meat infected with H5N1 HPAI virus produced systemic disease and was lethal [38]. Natural cases of H5N1 infection occurring after the consumption of infected birds also produced lesions of necrotizing hepatitis in large and small felids and dogs [12, 13, 35, 36], as well as lesions and virus in the myenteric plexus neurons of intestines of cats [15], suggesting possible digestive system exposure. However, such lesions could be the result of systemic virus dissemination after respiratory virus infection, with secondary infection of the digestive organs. The demonstration of lesions and viral antigen in the gastric or intestinal epithelium during the early phases of infection is critical to confirm the pathogenesis of infection in the digestive system.

The H5N1 HPAI virus can cause infection in carnivores and scavengers after consumption of infected carcasses or consumption of raw, uncooked, infected poultry by-products. However, this oral exposure is more likely to initiate infection through the respiratory system than through the digestive system. In humans, the potential for transmission of H5N1 HPAI virus is greater through inhalation or direct contact of oral, nasal, or conjunctival mucus membranes with contaminated fingers than through consumption of contaminated foods, because the vast majority of poultry is consumed as cooked product and is not consumed as raw or undercooked poultry products. Cooking is very effective at killing HPAI virus [39, 40].

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